IMPACTS OF STRESS AND DISTURBANCE ON BIOLOGICAL NITROGEN FIXATION AND TREE NUTRITION IN THE BOREAL FOREST

LES IMPACTS DU STRESS ET DES PERTURBATIONS SUR LA FIXATION BIOLOGIQUE DE L'AZOTE ET LA NUTRITION DES ARBRES EN FORÊT BORÉALE

par

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SUMMARY

This thesis deals with two important aspects that affect nutrition of the boreal forest: stress and disturbance. Stress in this context refers to the nutritional stress of plants defined as any amount of nutrient that decreases productivity. Disturbance refers to as any event that results in a significant loss of biomass in an ecosystem, thereby altering its structure. These interferences can be either detrimental or beneficial on tree growth, depending on forestry management.

In my first chapter, I examined roadside car emissions and their effects on tree nutrition and biological nitrogen fixation by moss-associated cyanobacteria. Experimental protocols included acetylene reduction assays to estimate biological nitrogen fixation of mosses (*Pleurozium schreberi*) and elemental analyses to measure nutrient and heavy metal concentrations in Norway spruce needles (*Picea abies*). We also investigated the effect of molybdenum and phosphorus additions on biological nitrogen fixation along a supposed gradient of atmospheric nitrogen deposition produced by exposure to roadside emissions. We observed a decrease in biological nitrogen fixation rates with increasing distance from the roadside. The effect of molybdenum and phosphorus on the biological nitrogen fixation was rather rare and showed no spatial patterns. We did not detect a nitrogen deposition gradient, but we found an increase in heavy metals and phosphorus in leaf tissue near roads, which may explain the decrease in nitrogen fixation rates.

My second chapter examines the effects of soil scarification, after clearcutting, on the nutritional quality of regeneration of black spruce (*Picea mariana*). More specifically, we evaluated whether scarification could reduce nitrogen and phosphorus deficiencies. Nutrient deficiencies were assessed by measuring changes in foliar nitrogen and phosphorus concentrations following fertilization. We used three approaches to derive the nutritional status of trees: (1) critical nutrient concentrations, (2) nitrogen / phosphorus ratios, or (3)

vector diagnostic analysis. We have shown that 18 years after scarification, nitrogen and phosphorus deficiencies have been reduced. At both sites, phosphorus was found to be more deficient than nitrogen. The effect of scarification was more beneficial in a maritime climate than in a continental climate.

Key words

Biological nitrogen fixation, Boreal forest, Disturbance, *Pleurozium schreberi, Picea abies, Picea mariana,* Scarification, Stress.

SOMMAIRE

Ce mémoire traite de deux aspects importants ayant une incidence sur la nutrition de la forêt boréale, soit le stress et la perturbation. Le stress dans ce contexte se réfère au stress nutritionnel des plantes défini comme toute quantité de nutriment qui diminue la productivité. La perturbation, quant à elle, désigne tout événement qui résulte en une perte considérable de biomasse vivante dans un écosystème, modifiant ainsi sa structure. Ces deux types d'interférences peuvent être préjudiciables ou bénéfiques pour la croissance des arbres, dépendant de la gestion forestière.

Dans mon premier chapitre, j'ai examiné les émissions des voitures en bordure de route et leurs effets sur la nutrition des arbres et sur la fixation biologique de l'azote par les cyanobactéries associées aux mousses. Les protocoles expérimentaux comprenaient des essais de réduction de l'acétylène pour estimer la fixation biologique de l'azote des mousses (*Pleurozium schreberi*) ainsi que des analyses élémentaires pour mesurer les concentrations de nutriments et de métaux lourds dans les aiguilles d'épinette de Norvège ainsi que dans les tissus de mousse. Nous nous sommes également intéressé à l'effet des ajouts de molybdène et de phosphore sur la fixation biologique de l'azote le long d'un supposé gradient de dépôt d'azote atmosphérique produit par l'exposition aux émissions en bordure de route. Nous avons observé une diminution des taux de fixation biologique de l'azote avec l'augmentation de la distance de la route. L'effet du molybdène et du phosphore sur la fixation biologique de l'azote était plutôt rare et ne démontrait aucun patron spatial. Nous n'avons pas décelé de gradient de dépôt d'azote, mais nous avons constaté une augmentation de métaux lourds et de phosphore dans les tissus foliaires près des routes, ce qui pourrait expliquer la diminution des taux de fixation biologique de l'azote.

Mon deuxième chapitre examine les effets du scarifiage du sol, après coupe à blanc, sur la qualité nutritionnelle de la régénération de l'épinette noire (*Picea mariana*). Plus

spécifiquement, nous avons vérifié si le scarifiage pouvait atténuer les carences en azote et en phosphore. Les carences en nutriments ont été évaluées en mesurant les changements des concentrations foliaires en azote et en phosphore suite à une fertilisation. Nous avons utilisé trois approches pour déduire le statut nutritionnel des arbres, soit (1) les concentrations critiques en éléments nutritifs, (2) les ratios azote / phosphore ou (3) l'analyse diagnostique vectorielle. Nous avons démontré que 18 ans après le scarifiage, les carences en azote et en phosphore ont été réduites. Sur les deux sites, le phosphore s'est révélé plus déficient que l'azote. L'effet du scarifiage était plus bénéfique dans un climat maritime que dans un climat continental.

Mots Clés

fixation biologique de l'azote, forêt boréale, perturbation, *Pleurozium schreberi, Picea abies, Picea mariana,* scarifiage, stress.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
μg	Microgram
Ag	Silver
ARR	Acetylene Reduction Rate
ATP	Adenosine Triphosphate
BNF	Biological Nitrogen Fixation
$Ca(H_2PO_4)_2$	Monocalcium Phosphate
Cd	Cadmium
cm	Centimeter
Co	Cobalt
Cr	Chromium
CRM	Certified Reference Material
Cu	Copper
dia.	Diameter
Fe	Iron
g	Gram
h	Hour
ha	Hectare
HNO_3	Nitric Acid
HSD	Honest Significant Difference
kg	Kilogram
km	Kilometer
L	Liter
m	Meter
mg	Milligram

mL	Millilitre
mm	Millimetre
Mo	Molybdenum
N_2	Di-nitrogen
N	Nitrogen
Na_2MoO_4	Sodium Molybdate
NaH_2PO_4	Monosodium Phosphate
ND	Nitrogen Deposition
NH_4NO_3	Ammonium Nitrate
Ni	Nickel
NPP	Net Primary Productivity
P	Phosphorus
Pb	Lead
QQ	Quantile-Quantile
RSD	Relative Standard Deviation
SE	Standard Error
SRM	Standard Reference Material
V	Vanadium
yr	Year
Zn	Zinc

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1. INTRODUCTION

The boreal forest forms a continuous belt across North America and Eurasia, making it one of the largest biomes on Earth. Collectively, it comprises 11% of the Earth's terrestrial surface and 33% of its forested area (Bonan and Shugart, 1989). This biome can store more carbon than any other biome (Ciais et al., 2008). Therefore, these boreal forests can significantly contribute to mitigating global environmental change, especially if their growth can be maintained. This level of growth can only occur when the detrimental effects of stress and disturbance are minimized. The stresses and disturbances imposed by anthropogenic influence will only continue expanding in northern hemispheres and nutrient cycling within this biome will need to be more thoroughly understood in order to optimize growth for both environmental and economic reasons (Lemprière et al., 2013).

In terrestrial boreal ecosystems nitrogen (N) is often the most limiting nutrient to net primary productivity (NPP) (Tamm, 1991). This is due to the low temperatures, acidic soil, recalcitrant nature of litter (Lambers et al., 2006). The incorporation of new forms of reactive N into these ecosystems is generally low. For example, their incorporation can occur in two main ways: 1) In areas of anthropogenic influence N enters the ecosystem mainly through the combustion of fossil fuels which are released into the atmosphere as nitrogen oxides then fall onto the biosphere via deposition processes. 2) In pristine boreal ecosystems N can enter the biosphere through biological nitrogen fixation (BNF).

Nitrogen fixation is the process of converting unreactive dinitrogen (N_2), which composes 78 % of the atmosphere, and fixing it via the nitrogenase enzyme into plant available forms. It is challenging to quantify the total amount of N entering boreal forests via this process due to temporal and spatial heterogeneity within ecosystems. Estimates have placed the boreal forest BNF contribution at 1.5 - 2.0 kg N ha^{-1}, compared to contributions in temperate forests at 6.5 - 26.6 kg N ha^{-1} (Cleveland et al., 1999). However, more recent estimates taking into account only contributions from *Pleurozium schreberi,* the most abundant boreal forest moss, estimate inputs at 1.7 kg N ha^{-1} (DeLuca et al., 2002). This estimation would double the estimate that had previously been calculated. A lack of intensive sampling may account for low estimates of BNF. Biomass of moss layers can at times even surpass the NPP of trees in their environment (Bond-Lamberty et al., 2007). The role that bryophytes play in regulating the nutrient cycling should be further evaluated.

Nitrogen deposition has increased three to five-fold over the last century (Denman et al., 2007), with global rates of deposition expected to increase by a factor of 2.5 by the end of this century (Lamarque et al., 2005). Nitrogen deposition occurs from inputs of reactive N from the atmosphere to the biosphere in gases, dry deposition, and as wet deposition in precipitation. This deposition results directly from global emissions of oxidized nitrogen from combustion of fossil fuels, and also reduced N from agricultural sources. Nitrogen cycling has been so extensively affected that more atmospheric N_2 is fixed by human activities than the combination of all natural processes (Vitousek et al., 1997).

Anthropogenic N inputs are particularly essential for boreal ecosystems with their characterization of N-limitation (Tamm, 1991). It has been recently observed that at least 71 % of the boreal forest receives N deposition rates at or below 3 kg N ha^{-1} yr^{-1} (Gundale et al., 2011). However higher nitrogen deposition in the range of >10 kg N ha^{-1} yr^{-1} can occur locally (Rousk and Michelsen, 2016). Deposition effects can depend on the critical load that

the system can tolerate. This refers to the highest deposition load not causing changes in soil chemistry leading to effects on the structure and function of the ecosystem (Ouimet et al., 2001). The ability of an ecosystem to resist these changes depends on: 1) the duration and total input 2) the sensitivity of plant species 3) abiotic factors including— acid neutralizing capacity, soil nutrient availability and also soil factors affecting nitrification potential and N immobilization rate (Bobbink et al., 2010). The level of N deposition that would cause nitrogen saturation in this ecosystem is up for debate. However, Gundale et al. (2011) showed that due to a 'bryophyte effect' the soil inorganic N availability and its acquisition by woody plants remained unchanged at levels of 12 kg N ha^{-1} yr^{-1}. This may not be consistent throughout all of the boreal forest and may change depending on the abundance of bryophytes. It has also been shown that as nitrogen sources increase there would be a decrease in total biomass of bryophytes (Gundale et al., 2011), this could act to create a feedback loop, where nitrogen deposition decreases bryophyte presence, subsequently less bryophytes are present to contain this nitrogen and therefore a lower level of deposition may cause negative environmental effects faster than what had been observed. Therefore, it is likely that we have not yet reached the critical level for N saturation in most boreal ecosystems. However, it is not likely that BNF and nitrogen deposition are additive. There have been many studies that have shown that BNF decreases as deposition of N increases (Rousk et al., 2013). Therefore, as N limitation on BNF is reduced other nutrients may become limiting or co-limiting.

The primary nutrient that has been long considered to become limiting to the BNF reaction is phosphorus (P) (Vitousek and Field, 1999; Vitousek et al., 2002). BNF is an energy intensive process requiring large amounts of adenosine triphosphate (ATP). BNF is one of the most metabolically costly processes in biology, 15 molecules of ATP consumed per molecule of fixed N_2 (Simpson and Burris, 1984). Another nutrient that has been shown to be limiting to BNF in forest ecosystems is molybdenum (Mo) (Silvester, 1989; Barron 2008). The nitrogenase enzyme requires Mo as a metal cofactor, and being the least abundant biologically required metal in the earth's crust, it is expected that Mo would be a limiting factor

(Wedepohl, 1995). Mo and P limitation as well as Mo-P co-limitation of asymbiotic N-fixation has been demonstrated in natural ecosystems and laboratory experiments (Barron et al., 2008; Jean et al., 2013; Wurzburger et al., 2012, Rousk et al., 2017). Mosses receive their nutrition from three sources: 1) throughfall deposition 2) recycling 3) passive uptake from soil. Throughfall being the main nutrition source of mosses, the increase of deposition rates will impact how these plants regulate their nutrient cycling. Therefore, investigation into how mosses respond to increased deposition inputs, particularly how the availability of essential nutrients like Mo and P will change should be quantified.

It should be noted that atmospheric deposition is a complex mixture of elements, not only N. Therefore, it is important to consider the entire makeup of deposition when looking at nutrient stress on the BNF process. For example, deposition particularly along roadsides can contain an array of heavy metals (i.e. Lead, Cadmium, Iron, Vanadium, Molybdenum) (Viskari et al., 1997; Figueira et al., 2002; Zechmeister et al., 2006). Vehicles that pass along these roads can contain various metals, therefore, the breakdown of their components, not only emissions need to be studied. Along these lines P is an element that exists as a non-exhaust source of emission from the wear of car parts like tires and brakes (Lim et al., 2006). This gives way to the ancillary objective in Chapter 1 of this project to evaluate not only Mo and P effects on BNF but also the effects of heavy metals and P deposition that may be occurring along roadsides.

Stress in the boreal forest is not limited to mosses. Trees will also be subjected to similar stresses as bryophytes occurring on the forest floor. In order to provide a more complete picture of nutrient cycling we should evaluate tree nutrient deficiencies as well. The foliage of forest trees can be excellent bio-indicators of ecosystem nutritional status, as tree nutrition is closely related to availability and concentration of nutrients in the soil (Tomlinson, 1991). Foliar analysis is the most widely accepted method to evaluate these nutrient deficiencies in

forests (Perry, 1994). A nutrient may be considered limiting if when added productivity increases (Gibson, 1971). Therefore, as originally noted by Liebig's law of minimum the nutrient that is in shortest supply will limit growth (Von Liebig, 1840). This idea gives way to the notion that nutrients are required in specific ratios, known as ecological stoichiometry. In northern ecosystems Nitrogen and phosphorus are often the two main limiting nutrients in forest productivity (Chapin, 1980).

In terrestrial ecosystems, a nitrogen to phosphorus ratio of 10:1 has been suggested and may be the optimal ratio, but this ratio may also depend on factors like species, growth rate, plant parts, and age (Gusewell, 2004; Knecht and Goransson, 2004). If these ratios were to be offset by the addition of a nutrient (i.e. Deposition) there would be repercussions toward plant growth. Therefore, the measure of these ratios may be one of the easiest and most direct ways to determine the presence of a nutrient imbalance (Koerselman & Meuleman, 1996). Through these methods we can also determine how disturbance is affecting cycling of N and P in boreal forest ecosystems, particularly those which are under silvicultural management.

Scarification is an economically important disturbance that is conducted as part of silvicultural management in boreal forests worldwide. Scarification is the process of disturbing the forest soil to create an optimal microsite and minimize competition from ericaceous shrubs allowing increased growth of the desired tree species. By exposing the mineral soil, scarification mimics conditions that would be present after a natural disturbance process, such as wind throw or fire events.

Scarification can control many ericaceous shrubs like *Kalmia* and *Rhododendron* (Thiffault and Jobidon, 2006). The control of these shrubs is economically important as they cause dense heaths that prevent trees from growing at their full potential either by direct interference

through competition for light and nutrients or by indirect interferences such as allelopathy (Thiffault and Jobidon, 2006). The growth check that *Kalmia* creates is due to the immobilization of N into a tannin-protein complex (Bradley et al., 1997). It has also been shown that ericaceous shrubs do not affect plant growth similarly in all climates (Yamasaki et al., 1998). Therefore, the control of this ericaceous shrub through scarification is particularly important on sites where silvicultural outcomes are desired.

Scarification has no significant effect on soil extractable N, however seedlings planted in scarified plots had higher foliar N concentrations compared to control plots (Timmer, 1991). Scarification also has a negative impact on soil extractable P, but this does not lead to lower foliar P concentrations (Thiffault and Jobidon, 2006). However, lower P concentrations in foliage have been found after scarification (Macdonald et al., 1998) and a reduction in available P was observed in scarified soils (Krause and Ramlal, 1987). It has been hypothesized that scarification may somehow favor nutrient absorption, possibly through increased root growth. It has also been shown that scarification has negative impacts on interactions of site nutrients including leaching of cations due to increased nitrification (Vitousek et al., 1992, Munson et al., 1993). Therefore, it is well established that scarification can have varying impacts on nutrient cycling in boreal forests. However, the extent to which scarification affects nutrient cycling of N and P, allowing trees to express their full growth potential is still not clear.

The present study seeks to understand how stress and disturbance will impact nutrient cycling in the boreal forest. The first objective was to verify how Mo and P addition would affect BNF along an assumed gradient of atmospheric deposition, causing nutrient stress to the moss-associated BNF. The second objective was to determine the impact of soil scarification on N and P nutrition of black spruce trees in two climatic regimes.

2. CHAPTER 1

Anthropogenic roadside deposition of nitrogen, phosphorus, and heavy metals stresses nutrient cycling by moss-associated cyanobacteria and trees in Northern Sweden.

Reference: Anthropogenic deposition of heavy metals and phosphorus may reduce biological N_2 fixation in boreal forest mosses. Science of the Total Environment (Scott et al., 2018)

This manuscript describes a study where we measured the effect of roadside automobile emissions on biological nitrogen fixation of boreal forest moss-associated cyanobacteria in Northern Sweden. Biological nitrogen fixation in boreal mosses had previously been shown to be an important mechanism by which reactive nitrogen is incorporated into pristine northern ecosystems. Our initial hypotheses focused on how molybdenum and phosphorus additions would influence biological nitrogen fixation along an assumed atmospheric nitrogen deposition gradient created by exposure to roadside emissions.

Our results revealed decreasing biological nitrogen fixation with roadside distance, but no evidence of a nitrogen deposition gradient as indicated by moss tissue nitrogen. Instead, we found some evidence that decreasing biological nitrogen fixation rates result from heavy metal and phosphorus emissions from cars. The implications of our study are that anthropogenic heavy metal and phosphorus deposition may have unsuspected and substantial effects on the terrestrial nitrogen cycle in northern latitudes.

Anthropogenic deposition of heavy metals and phosphorus may reduce biological N_2 fixation in boreal forest mosses.

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2.1.Abstract

A study was undertaken to test the effects of molybdenum (Mo) and phosphorus (P) amendments on biological nitrogen (N) fixation (BNF) by boreal forest moss-associated cyanobacteria. Feather moss (*Pleurozium schreberi*) samples were collected on five sites, on two dates and at different roadside distances (0–100 m) corresponding to an assumed gradient of reactive N deposition. Potential BNF of Mo and P amended moss samples was measured using the acetylene reduction assay. Total N, P and heavy metal concentrations of mosses collected at 0 and 100 m from roadsides were also measured. Likewise, the needles from Norway spruce trees (*Picea abies*) at different roadside distances were collected in late summer and analyzed for total N, P and heavy metals. There was a significant increase in BNF with roadside distance on 7-of-10 individual Site × Date combinations. We found no clear evidence of an N gradient across roadside distances. Elemental analyses of feather moss and Norway spruce needle tissues suggested decreasing deposition of heavy metals (Mo-Co-Cr-Ni-V-Pb-Ag-Cu) as well as P with in- creasing distance from the roadside. The effects of Mo and P amendments on BNF were infrequent and inconsistent across roadside distances and across sites. One particular site, however, displayed greater concentrations of heavy metals near the roadside, as well as a steeper P fertility gradient with roadside distance, than the other sites. Here, BNF increased with roadside distance only when moss samples were amended with P. Also at this site, BNF across all roadside distances was higher when mosses were amended with both Mo and P, suggesting a co-limitation of these two nutrients in controlling BNF. In summary, our study showed a potential for car emissions to increase heavy metals and P along roadsides and underscored the putative roles of these anthropogenic pollutants on BNF in northern latitudes.

Keywords

Boreal forest; Car emissions; N fixing cyanobacteria; *Pleurozium schreberi; Picea abies*

2.2. Introduction

Biological nitrogen fixation (BNF) by moss-associated cyanobacteria can be an important source of reactive N into boreal forests (DeLuca et al., 2002). Accordingly, several studies over the past decade have focused on the environmental and biochemical factors controlling this process. For example, studies have found positive relationships between BNF and moisture in moss carpets (e.g. Gundale et al., 2009; Jackson et al., 2010), as well as optimal ranges for temperature and light intensity (Gundale et al., 2012). Likewise, studies have found a negative relationship between BNF and nitrogen (N) availability in moss carpets, either as a result of incremental rates of fertilizer N additions (Gundale et al., 2011) or gradients of throughfall N (DeLuca et al., 2008; Rousk et al., 2013). More recently, studies on BNF have probed the putative roles of molybdenum (Mo), which is an essential co-factor of the nitrogenase enzyme complex, or phosphorus (P), which is required for large ATP expenditure (e.g. Jean et al., 2013; Rousk et al., 2017). These studies on Mo and P limitations have yielded inconsistent and sometimes opposing results, suggesting that BNF in boreal moss carpets could be regulated by interactions between several nutrients, such as N, P and Mo, as well as other elements. A potentially powerful approach that might help reveal how elements interact to control BNF is to study the effects of P and Mo availability, as well as other elements, along a gradient of atmospheric N deposition.

It is known that steep N deposition gradients may occur along roadsides, where car exhaust emissions carry various forms of reactive nitrogen (e.g. Bignal et al., 2007; Redling et al., 2013). Ackermann et al. (2012) reported lower BNF rates in feather mosses (*Pleurozium schreberi* (Brid.) Mitt.) adjacent to busy roads compared to feather mosses along remote roads in Northern Sweden. Likewise, they reported increasing BNF rates with increasing roadside distance (0–100 m) along busy roads, but not along remote roads where BNF was consistently

high. That lack of BNF gradients near remote roads confirmed that BNF gradients near busy roads were not a forest gap effect. They concluded that BNF rates in feather mosses represent a highly sensitive indicator for anthropogenic N pollution to natural systems. Thus, the busy road sites that they used are an ideal setting for testing the effects of Mo and P additions on BNF rates along a gradient of anthropogenic N deposition.

Ackermann et al.'s (2012) paper is an important contribution to our understanding of the biogeochemical controls on BNF in boreal forest mosses. Gradient studies like these, however, can often exhibit strong co-variation among plant nutrients, which may interactively influence BNF. For example, while there was a clear difference of N deposition between busy and remote roads, there was no apparent effect of roadside distance on N deposition along busy roads. By contrast, they found decreasing concentrations of zinc (Zn) and lead (Pb) in soil samples with increasing roadside distance, suggesting that the overall heavy metal load may be higher near roadsides. This is corroborated by several studies that have shown high heavy metal concentrations in moss tissues along roadsides (e.g. Pearson et al., 2000; Zechmeister et al., 2006). The effect of N deposition from car emissions may thus be confounded with the effect of other contaminants, as previous studies have shown a decrease in cyanobacterial BNF in soils contaminated with heavy metals (e.g. Lorenz et al., 1992). Car emissions also include vanadium (V), iron (Fe) and Mo (Lim et al., 2006), all three of which are alternative co-factors of nitrogenase enzyme systems. Phosphorus is a component of car emissions as well (Lim et al., 2006) and may also be released from the degradation of tires and brakes (Hulskotte et al., 2014). For these reasons, a study that tests the effects of car emissions on BNF should include indices of heavy metal deposition and N-to-P limitations as a function of roadside distance.

We report on a study where we tested the effects of P and Mo additions on BNF in feather moss carpets, at various distances from the same busy roads used by Ackermann et al. (2012). We hypothesized that N deficiency was an overriding condition for BNF to occur, and thus

predicted that Mo and P additions would increase BNF with increasing roadside distance. We also tested for roadside gradients of heavy metals and N-to-P limitations, based on elemental analyses of moss tissues and Norway spruce (*Picea abies* (L.) Karst.) needles.

2. 3. Materials and methods

2.3.1. Study sites

The field experiment was performed along roadsides at five sites located within a 50 km radius of the town of Arvidsjaur, in Northern Sweden (65°35′N 19°10′E). According to Ackermann et al. (2012), 450 to 1150 cars per day travel along these roads. The five sites are referred to as Arvidsjaur (65°58′N, 19°40′E), Borup (65°00′N, 19°25′E), Nyvall (65°23′N, 19°26′E), Strömsforsheden (65°08′N, 18°51′E) and Vilan (64°30′N, 18°52′E). Four of the five sites were the same as those used by Ackermann et al. (2012). Mean annual temperature at the five sites is 1 °C and mean annual precipitation is 570 mm. As reported by Ackermann et al. (2012), four of the sites were within forest reserves and had received no fertilization or timber harvest. Although information was not available for the fifth site (i.e. Arvidsjaur), there was no indication of recent logging activity at this site as well. All sites were comprised of mid- to late-successional forest stands dominated by Norway spruce, except the Strömsforsheden site, which was dominated by Scots pine (*Pinus sylvestris* L.). The soils along all road sites can be classified as Haplocryods (Soil Survey Staff, 1999).

2.3.2. Sampling mosses and needles

In late-May 2016, we established four long and narrow rectangular plots (2 m × 100 m) at each of the five sites. The four plots were oriented parallel to the roadside, at four distances (0, 10, 50 and 100 m) from the road. The 0 m distance was established at the forest edge, which varied between 5 and 10 m from the roadside across sites. Red-stemmed feathermoss (*Pleurozium schreberi* (Brid.) Mitt.) stems were collected along the entire length of each plot to fill a 7.5 L plastic bag. The 20 bulked moss samples (i.e. 4 plots × 5 sites) were then transported to the laboratory (i.e. Swedish University of Agricultural Sciences, Umeå, Sweden) for further analyses (see below). The same procedure was repeated in mid-September 2016. Also in mid-September, we counted the number of moss stems occurring in three randomly positioned metal cylinders (10 cm dia.) in each plot.

In mid-September 2016, the needles of six to nine trees were sampled at each of the four sites dominated by Norway spruce (N = 27). The trees were selected to cover the 0–100 m range in roadside distance. The height of the chosen trees (7–11 m) was fairly uniform within sites (avg. RSD = 23%) and differed mainly across sites. Three branches were harvested around the top third of each tree and first- year needles were removed and pooled together. The 27 needle samples were transported to the laboratory where they were dried (60 °C, 24 h) and stored until further analyses (see below).

2.3.3. Assessing the effects of Mo and P on acetylene reduction rates

Potential BNF rates were estimated using the acetylene reduction assay (Stewart et al., 1967). At each sampling date, each bulked moss sample was gently mixed and ten randomly selected stems, excised at 5 cm, were placed top-down into each of 32 glass vials (20 mL). We then sprayed 4 mL of aqueous Mo (4×10^{-5} g L^{-1} as Na_2MoO_4), P (1.4×10^{-3} g L^{-1} as NaH_2PO_4) or Mo + P (same concentrations) solution in each of eight vials. Deionized ultra-pure water was applied in each of the remaining eight vials. These four treatments are hereafter referred to as +Mo, +P, +Mo + P, and Control. The 640 glass vials containing amended mosses were then inverted, placed in closed-bottom polystyrene foam trays and transferred to an incubator set at 18 °C with light intensity set at 500 μmol m^{-2} s^{-1}. After 24 h, we crimped an air-tight lid equipped with a rubber septum, we removed 3 mL of headspace gas and injected 3 mL of acetylene (C_2H_2) into each vial. At this stage, we added three types of controls: (1) 60 vials containing only C_2H_2 to check for the degradation of acetylene to ethylene, (2) 40 vials containing only C_2H_4 to be used as ethylene standards, and (3) 60 vials containing only water-amended mosses (i.e. three vials from each plot) to check for possible natural ethylene production of mosses. The 800 vials (per sampling date) were incubated for another 24 h at 18 °C with light intensity at 500 μmol m^{-2} s^{-1}. The headspace gas in each vial was then analyzed for ethylene concentration using a Perkin Elmer Clarus 500 with TurboMatrix 40 headspace injector (Perkin Elmer, Waltham, Massachusetts, USA). Following the assay, mosses were dried at 60 °C for 24 h to determine dry biomass in each vial. Acetylene reduction rates (ARR) were reported on a moss mass basis. The dried moss samples from the water-amended Control treatment were then used for elemental analyses (described below).

2.3.4. Moss and needle elemental analyses

Moss samples from the 0 and 100 m distances, and Norway spruce needles collected across roadside distances, were analyzed for their elemental concentrations. More specifically, dried moss and needle samples were mixed with liquid N_2 and ground into a powder using a mortar and pestle. Ground subsamples (ca. 100 mg) from each moss vial, and triplicate needle subsamples from each tree, were encapsulated and analyzed for total N using a Vario Macro Elemental Analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). For each of the 10 plots (5 sites × 2 roadside distances), the remaining ground moss was pooled into three separate samples. These, along with needle samples from each tree, were digested in trace metal grade HNO_3 and diluted with MilliQ water. The digests were then analyzed for total P and heavy metals (Co, Cr, Ni, V, Pb, As, Cu, Mo) using an X-Series-2 Inductively Coupled Plasma–Mass Spectrometer (Thermo Scientific, Waltham, Massachusetts) (Rodushkin et al., 1999). The reference materials used for quality control were pine needles SRM 1575a (National Institute Standardized Testing, Gaithersburg, Maryland) and the certified reference material SLRS-6 (National Research Council of Canada, Ottawa, Canada).

2.3.5. Statistical analyses

The effect of roadside distance on moss stem density, as well as the effects of roadside distance, amendments (and their interaction) on ARR, were evaluated using mixed effects models with site as a random effects variable. Likewise, the effects of roadside distance and amendments (and their interactions) on ARR within each site were tested using fixed effects models followed by post hoc Tukey tests. For these models, individual moss samples (i.e. glass vials) were treated as true replicates, given that statistical inference was confined to within each site. We also compared acetylene reduction rates "near" (i.e. 0 and 10 m) and

"far" (50 and 100 m) from the roadside using single degree of freedom orthogonal contrasts. For each site, the effects of roadside distance (0 vs. 100 m) on total N, total P and heavy metal concentrations in mosses were determined using Student's t-test. Conversely, we assessed the effect of sites on total N, P and heavy metals within each roadside distance using Tukey's HSD test. We tested the effects of road- side distance on total-N, total-P, N:P ratios and heavy metal concentrations of Norway spruce needles using simple linear and exponential regression models.

Prior to analyses, all data were evaluated for normality and homoscedasticity respectively, using QQ-plots and the residuals vs. fitted values plots. If the data did not meet these assumptions, they were log-transformed. The data were analyzed using the lme4 (Bates et al., 2015) and stats packages of R statistical software (R version 3.3.2, R Core Team, Vienna, Austria). Unless otherwise stated, a significance level of 0.05 was used.

2.4. Results

2.4.1 Acetylene reduction rates

The mass of moss stems at each site increased with roadside distance while the density of moss stems did not. Consequently, ARR values that we report do not reflect potential BNF per ground surface area but rather BNF per moss mass. At each sampling date, ARR of non-amended mosses increased significantly ($P < 0.01$) with increasing roadside distance. This was

true when all five sites were pooled together (Fig. 1), as well as on 7-of-10 individual Site x Date combinations. The effects of Mo and P amendments on ARR across sites, and across roadside distances, were not significant. At Borup, however, the +P amendment increased (P = 0.02) ARR in mosses that were far (50–100 m) from the roadside, but not in mosses that were near (0–10 m) the roadside (Fig. 2). Also at Borup, ARR across all four roadsides distances was higher (P = 0.03) in the +Mo + P than in the other amendments (Fig. 3).

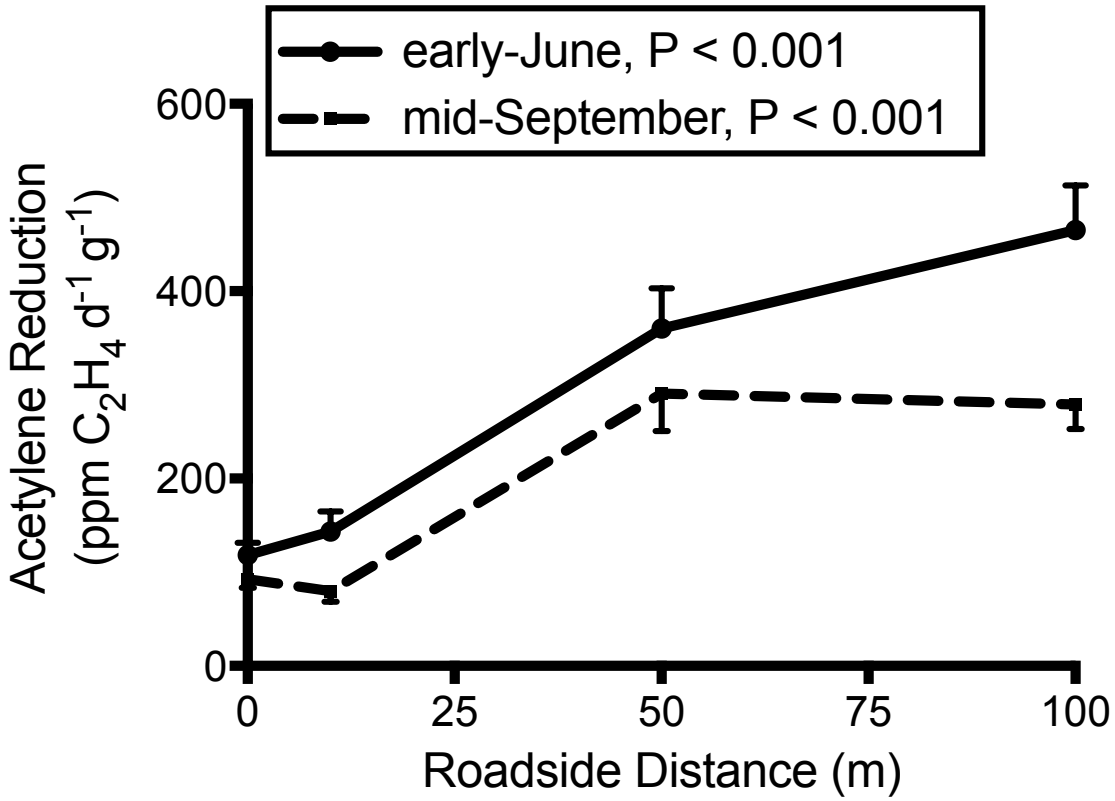

Fig. 1. Acetylene reduction rates of non-amended moss samples collected in early and late summer 2016, at four roadside distances (0, 10, 50 and 100 m) across five independent sites in Northern Sweden. The data were pooled across sites because similar trends were observed at all sites. P values are the result of mixed effects models testing the effect of roadside distance with site identity as a random effects variable (vertical lines = 1 S.E.; N = 160 per sampling date).

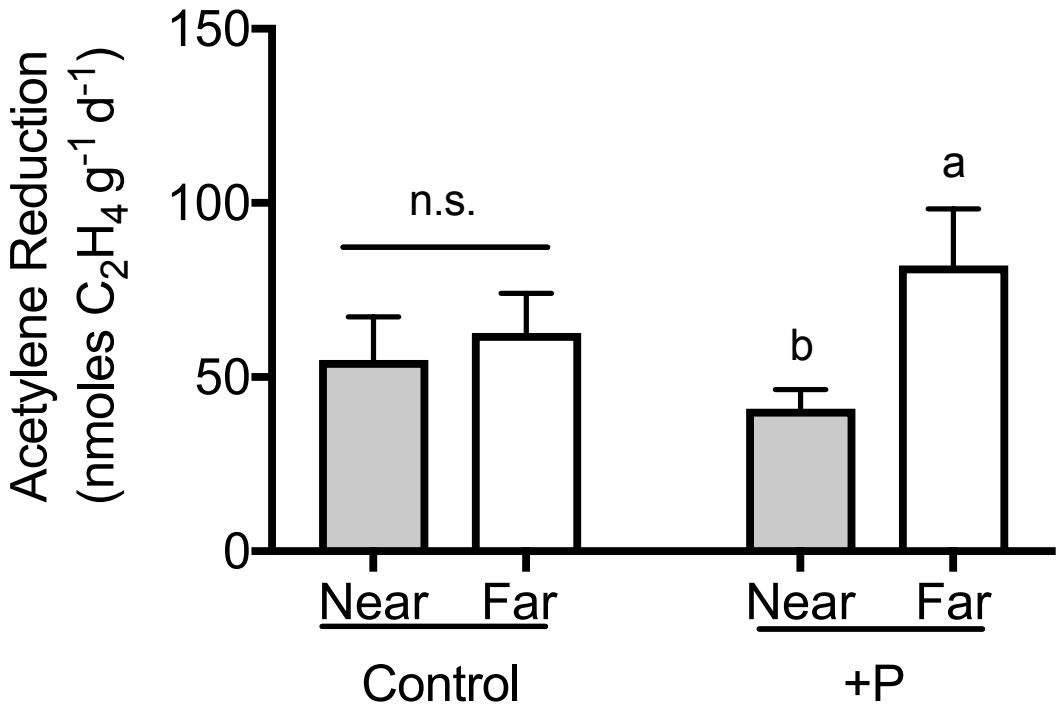

Fig. 2. Acetylene reduction rates of +P amended and non-amended (i.e. control) mosses collected near (0 and 10 m) and far (50 and 100 m) from the roadside at Borup. Values were pooled across both sampling dates because sampling date had no significant effect. Different lowercase letters designate a significant ($P < 0.05$) roadside distance effect based on single degree of freedom orthogonal contrasts (vertical lines = 1 S.E.; N=128).

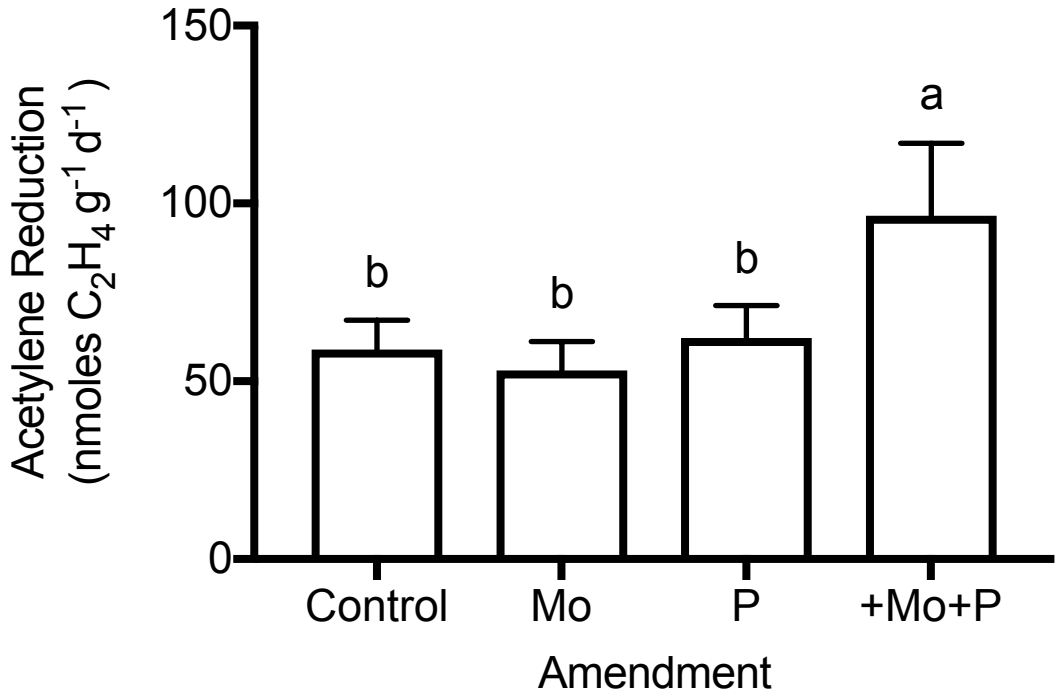

Fig. 3. The main effects of P, Mo and Mo + P amendments on BNF rates at Borup. Values were pooled across sampling dates and across roadside distances, as the effects of Mo and P amendments on ARR were not significant across these two experimental factors. Different lowercase letters designate significantly ($P < 0.05$) different means based on Tukey's HSD test (vertical lines = 1 S.E.; N = 256).

2.4.2. Elemental concentrations of mosses and needles

In four of the five sites, the concentration of heavy metals in mosses was consistently higher at 0 m than at 100 m from the roadside (Fig. 4). These differences were mainly driven by the concentrations of Cu, Pb, V, Ni and Cr. At the 100 m roadside distance, there were no significant differences in the heavy metal concentration of mosses between sites (8–11 μg g^{-1}). However, the total heavy metal concentration of mosses near the roadside (0 m) was significantly higher at Borup (54 μg g^{-1}) than at the other four sites (7–22 μg g^{-1}) (Fig. 4). At the Arvidsjaur site, moss N concentration was lower ($P = 0.02$) at 0 m than at 100 m from the roadside, while the opposite was true at the Strömsforsheden and Vilan sites ($P = 0.04$ and $P < 0.01$ respectively) (Fig. 5A). There was no effect of roadside distance on moss N concentration at both the Borup and Nyvall sites. In contrast, the P concentration of mosses was consistently higher ($P \leq 0.02$) at 0 m than at 100 m from the roadside in each of the five sites (Fig. 5B).

At Borup only, the total heavy metal concentration of Norway spruce needles decreased significantly ($P < 0.01$) with roadside distance. An exponential model provided the best fit for three of these elements (Pb, V, As), whereas a linear model provided the best fit for the other three elements (Mo, Cu, Ni) (Fig. 6). Across all sites, needle N and P concentrations ranged between 8.9 and 14.7 and 1.4–2.9 mg g^{-1} respectively. There was no significant effect of roadside distance on needle N concentration (data not shown). Needle P concentration decreased ($P < 0.01$), whereas the needle N:P ratio increased ($P = 0.02$), with increasing roadside distance (Fig. 7A). These patterns were mainly driven, however, by needles collected at Borup (Fig. 7B).

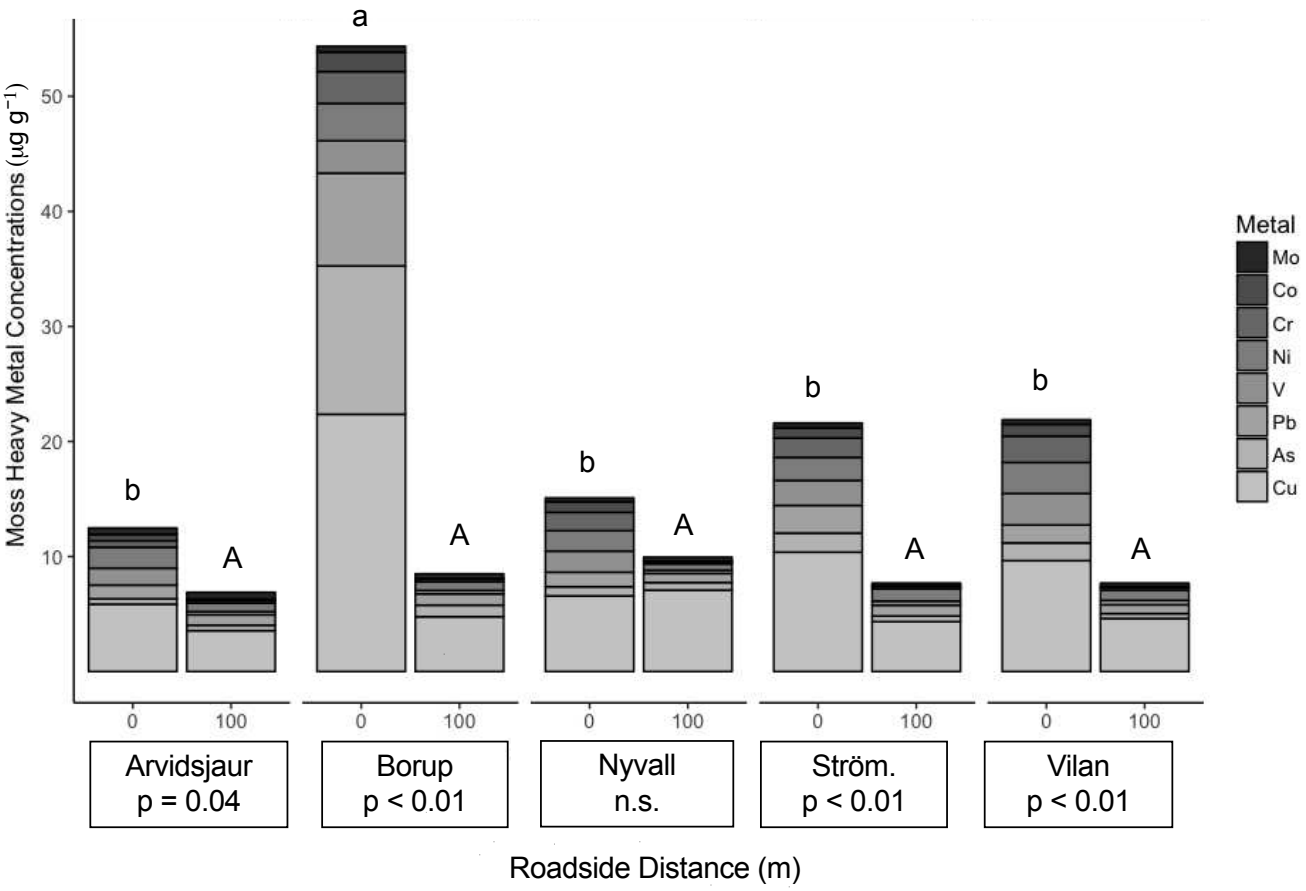

Fig. 4. Moss heavy metal concentrations at 0 m and 100 m distance from the roadside, at each site. The significance of roadside distance within each site (Student's t-tests) is shown in boxes below the abscissa. Different lower-case and upper-case letters denote significant differences ($P < 0.05$, Tukey's HSD tests) between sites, at the 0 m and 100 m distances respectively (average RSD = 0.21; N = 30).

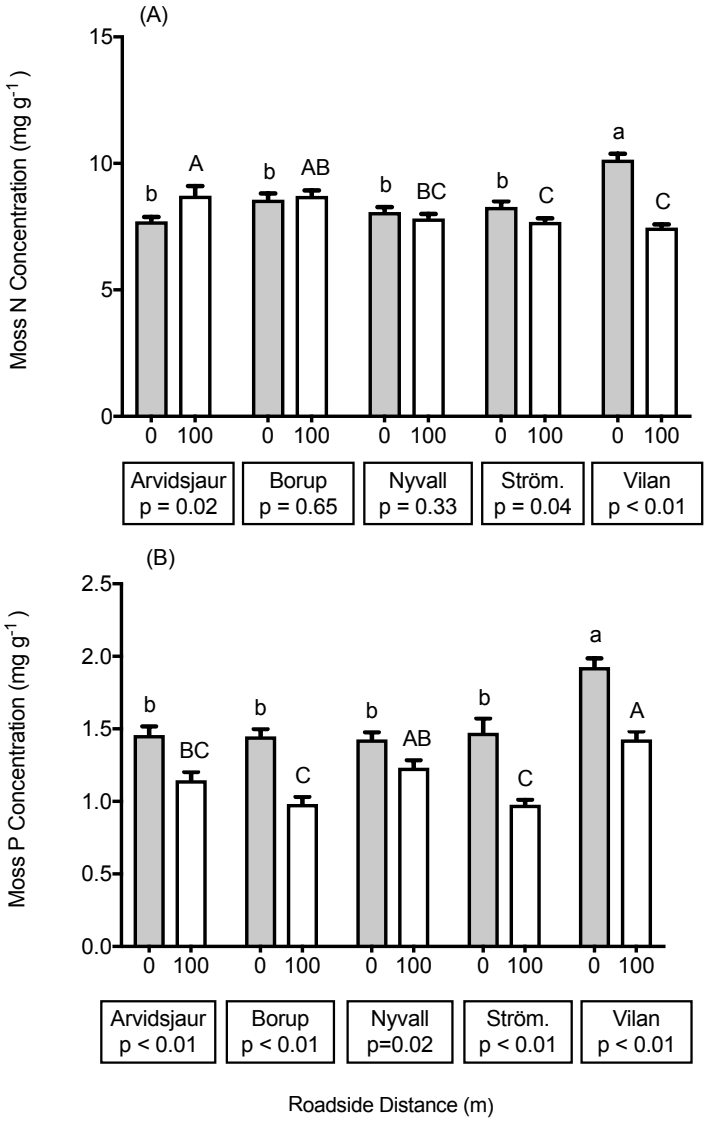

Fig. 5. Moss N (A) and P (B) concentrations at 0 m and 100 m distance from the roadside, at each site. Data are pooled across sampling dates. The significance of roadside distance within each site (Student's t-tests) is shown in boxes below the abscissa. Different lower-case and upper-case letters denote significant differences ($P < 0.05$, Tukey's HSD tests) between sites, at the 0 m and 100 m distances respectively (vertical lines = 1 S.E.; for nitrogen N = 160; for phosphorus N = 60).

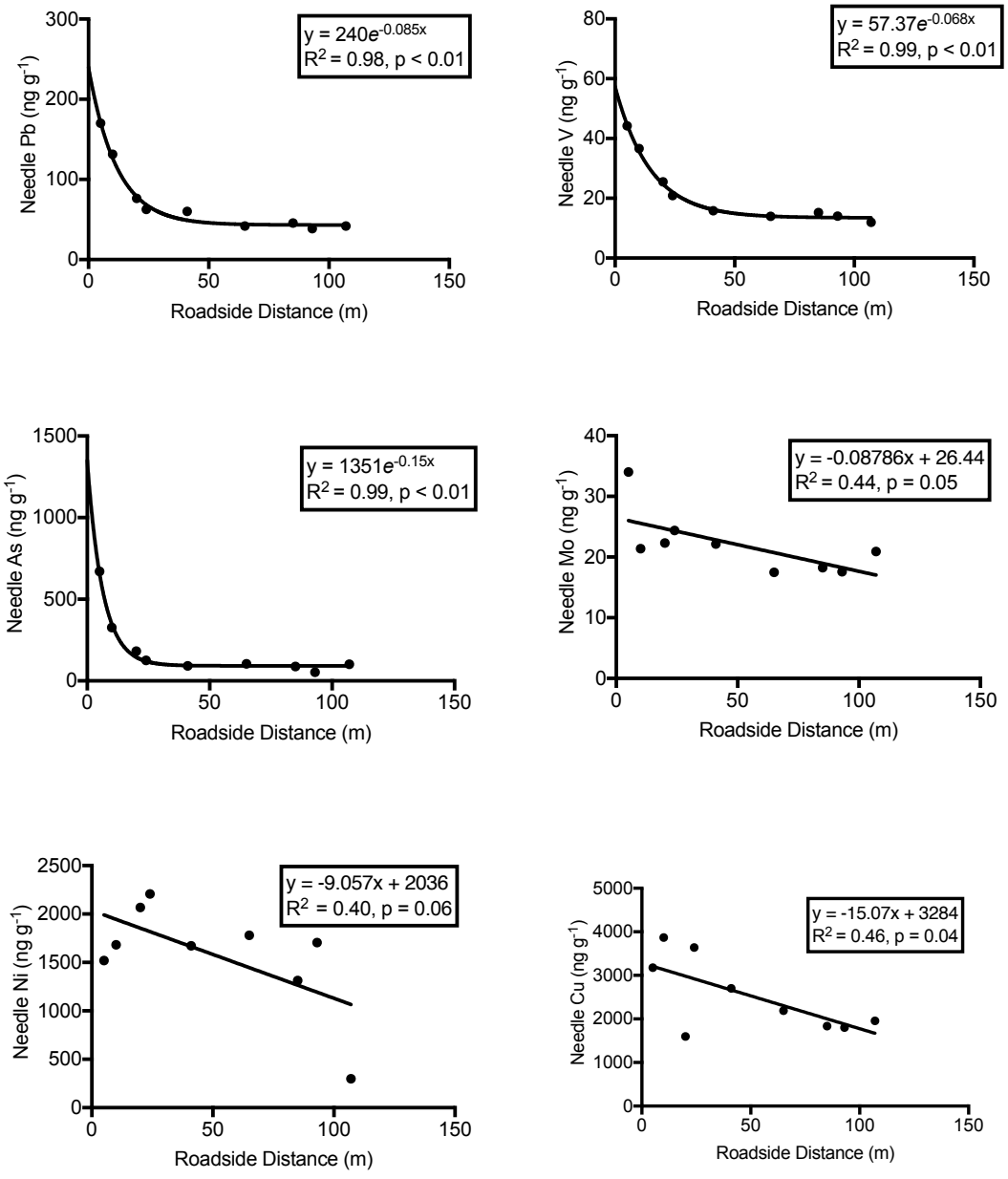

Fig. 6. Significant relationships between roadside distance and Norway spruce needle concentrations of six heavy metal of needles from unfertilized control trees at Borup. Results from the best-fitting simple linear or exponential regression models are shown in boxes within each frame.

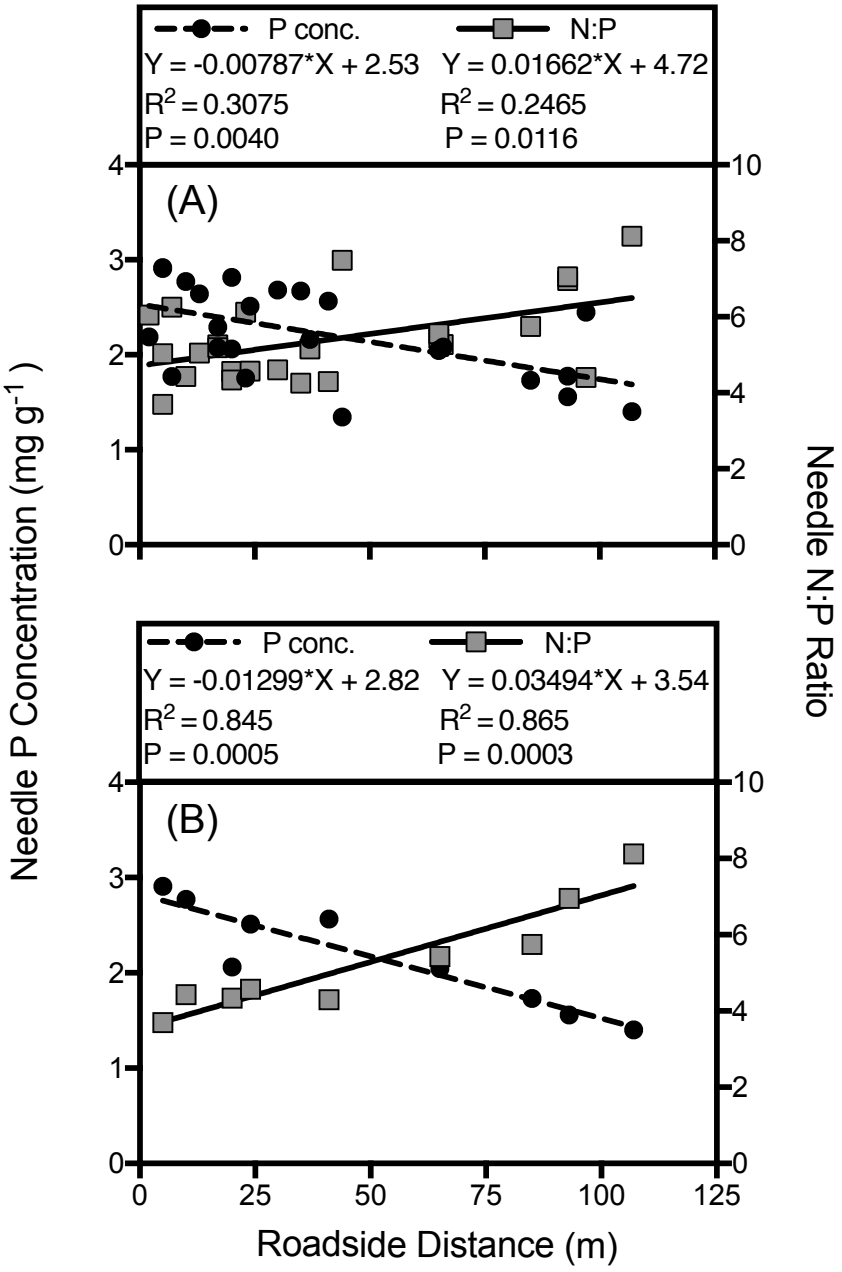

Fig. 7. The effect of roadside distance on needle P concentrations and N:P ratios of unfertilized trees (A) across all five sites and (B) at Borup only. Lines represent the best fitting linear regression models. Results from simple linear regressions are shown in boxes above each plot.

2.5. Discussion

Our results corroborate those from Ackermann et al. (2012), showing decreasing BNF rates by *P. schreberi* moss-associated cyanobacteria within 100 m of roadsides. The fact that this pattern was observed across five independent sites and on two sampling dates suggests that roadside BNF gradients may be prevalent across a broad range of Northern ecosystems. However, we found no clear evidence that these gradients resulted from gradients in anthropogenic N deposition. For instance, we found no consistent pattern in the N concentration of mosses and spruce needles across the five sites. This is in accordance with data presented by Ackermann et al. (2012) who were not able to detect a roadside distance effect on resin-sorbed N within throughfall collectors, nor on soil N concentrations, at the same sites. Collectively, these observations suggest that other compounds released from car emissions may play a role in decreasing BNF rates along these five roadsides.

Lim et al. (2006) listed 37 elements that were emitted from conventional car exhaust, which included the eight heavy metals measured in our study. There is good evidence in the literature that these elements may accumulate in moss tissues. For example, Sucharova and Suchara (2004) correlated heavy metal concentrations in *P. schreberi* to atmospheric deposition patterns across the Czech Republic. On a finer scale, Pearson et al. (2000) showed that moss tissue concentrations were good bio-indicators of heavy metal deposition from roadside car emissions. Heavy metals such as Zn, Cd, Ni and Cu have all been linked to a decrease in N-fixation activity by symbiotic Rhizobium spp. associated with legumes (Vesper and Weidensaul, 1978; Giller et al., 2009; Marino et al., 2013). Likewise, studies have shown inhibitory effects of Hg, As and Cu on N-fixation by asymbiotic N-fixers in compost and soils (Keeling and Cater, 1998; Oliveira and Pampulha, 2006). Studies specific to cyanobacteria

have also shown an inhibition of N-fixation in soils contaminated with Zn, Cu, Ni, Cd, Cr and Pb, sometimes at concentrations close to permissible limits (Brookes et al., 1986; Lorenz et al., 1992; Suroz and Palinska, 2004). Based on these numerous studies, and on the heavy metal concentrations we observed in moss and needle tissues, we posit that roadside BNF gradients could, at least in part, be due to heavy metal deposition from car emissions. Given that boreal forests comprise the world's largest biome, that *P. schreberi* is the most common boreal forest moss species (Kuc, 1997), and that human activities are steadily moving northward, we suggest that further research pay attention to the role that heavy metal deposition in Northern ecosystems may have on N cycling.

BNF is catalyzed by the enzyme nitrogenase, for which Mo is a cofactor. As Mo is the rarest bio-element in the earth's crust (0.00011%), the concentrations that we found in mosses and spruce needles were relatively low. On the other hand, nitrogenase is known to have two alternative isoforms based on vanadium (V) and iron (Fe). Compared to Mo, V concentrations of mosses and spruce needles were relatively high and consistently higher near the road than at 100 m distance. We might expect V deposition would stimulate BNF near the road, but the opposite was observed. This suggests that BNF was inhibited by heavy metals or P, regardless of whether the V nitrogenase isoform was present or not. In our study, we also measured Fe concentrations and found the same roadside gradients as the other heavy metals (data not shown). However, Fe concentrations in needles and mosses were two orders of magnitude higher than the other metals we measured. This is consistent with the fact that Fe is relatively abundant in the earth's crust (6.3%) as well as an important component of automobiles. Therefore, we assume that Fe was not a limiting nutrient for nitrogenase activity across the entire roadside gradient.

Heavy metal deposition may not only decrease BNF rates, but may also shift the uptake rates of various macronutrients. For example, Cu contamination was shown to increase P concentration in *P. schreberi* moss tissues (Kapusta and Godzik, 2013). In our study, roadside P gradients in moss and tree tissues may also be the direct result of P emissions from car exhaust (Lim et al., 2006). Accordingly, at each site we found higher moss P concentrations at 0 m than at 100 m from the roadside. Similarly, we found decreasing P concentrations in Norway spruce needles with increasing roadside distance across all sites, especially at Borup. The role that P might play in controlling BNF in various ecosystems has been discussed for some time (e.g. Vitousek and Howarth, 1991), but a general consensus has yet to emerge. For example, Chapin et al. (1991) showed a positive effect of P on BNF in Arctic soil cores. On the other hand, Jean et al. (2013) and Rousk et al. (2017) both found seasonal patterns in P limitation or P suppression of BNF, in various northern forest ecosystems. Finally, Barron et al. (2011) and Zackrisson et al. (2009) both found no evidence of P limiting BNF, in either tropical or boreal forest soils. Collectively, these past studies hint at complex interactions between factors such as climate, geological parent material, organic matter and other plant nutrients in determining the effect of P on BNF. At Borup, where the gradient in needle P and N:P ratios were the steepest, mosses far from the roadside had higher BNF rates than those near the roadside when amended with P. It is, however, difficult to conclude whether these P amendments suppressed BNF near the roadside, or stimulated BNF far from the roadside.

One of the interesting findings at Borup was the overall positive effect of +Mo + P amendments on BNF rates. Similar results were reported in a few studies (Barron et al., 2011; Wurzburger et al., 2012) where the authors raised the prospective explanation of nutrient co-limitation. While nutrient co-limitation contravenes the classic view of Liebig's Law of the Minimum (Liebig, 1842), more recent work has reviewed the various theories and the scientific evidence that support multiple-resource limitation in plants (Harpole et al., 2011). These authors point out the various cases where multiple nutrient limitations occur (i.e.

simultaneous, independent, synergistic and serial limitations), which do not all meet the strict definition of co-limitation (Craine and Jackson, 2010). Bloom et al. (1985) suggested that some plants may adjust their growth pattern so as to be limited by several resources simultaneously. As Mo is the least abundant of all biometals in the earth's crust, the uptake of Mo by cyanobacteria might be regulated by the production of specialized metallophores, as was shown for free- living N-fixing soil bacteria (Kraepiel et al., 2009). In keeping with the co-limitation concept of Bloom et al. (1985), moss associated cyanobacteria at Borup may be regulating Mo uptake only to maintain an optimal stoichiometric ratio with P.

The concept of critical nutrient concentrations for plant growth has been studied for a long time (e.g. Ingestad, 1962; Nihlgard, 1990). N and P concentration in Norway spruce needles may thus inform us on the relative deficiencies or surpluses of these two nutrients across experimental sites. For example, Brække and Salih (2002) reported critical needle N and P concentrations of >1.8% and >0.18% for Norway spruce. Based on these numbers, we surmise that all sites were N deficient but had surplus P. Many researchers agree, however, that needle N:P ratios are more informative on the relative limitation of each of these nutrients. Thus, for Norway spruce needles, optimal N:P ratios (g g^{-1}) were reported at around 6–7 by Clarholm and Rosengren-Brinck (1995), and at around 6–12 by Mellert and Göttlein (2012). Based on these numbers, we surmise that needle N:P ratios at Borup are suboptimal near the roadside (~4) and attain the optimal range (6–8) at around 100 m. The degree to which higher BNF far from roadsides is responsible for optimizing needle N:P ratios remains unclear, however, as recently fixed N_2 is strongly retained by moss tissues rather than transferred to neighboring trees (Houle and Moore, 2008; Rousk et al., 2016).

It may be argued that differences in BNF rates with increasing roadside distance might be due to forest edge effects resulting in differences in humidity or light intensity. Both of these environmental factors may affect BNF in moss associated cyanobacteria (Gundale et al. 2009 and 2012). We have reason to believe, however, that this was not the case. At the time of sampling, we took 25 hemispheric photos of the canopy at each site. These were digitally analyzed for canopy openness. Statistical analyses revealed that there were no differences in canopy openness between sites or along roadside distances (data not shown). This is because the roads are relatively narrow (i.e. 2 cars width) whereas forest stand density is relatively low. Likewise, if there were humidity gradients, these would probably not correlate with roadside distance as much as with topography (i.e. drainage). Two of our sites were flat, two of our sites had a slight downslope and one site had an upslope. Thus, gradients in drainage with increasing roadside distance probably differed across the five sites. In spite of this, we were able to extract generalizable trends in BNF, which supports the interpretation of our data based on the observed trends in P and heavy metal deposition.

2.6. Conclusions

Our results show a 2–3 fold increase in moss-associated N-fixation along 100 m distances from roadsides in Northern Sweden. While we expected that N inputs from car emissions would be driving these BNF gradients, our results suggest alternative factors. More specifically, we show evidence that heavy metals and P from car exhausts play important roles in controlling moss-associated N-fixation and possibly tree growth. Furthermore, Mo may also limit BNF, but its relationship to the P status of mosses remains unclear. Thus, future research should strive to understand (1) the relative contributions of N, P, Mo and specific heavy metals on moss-associated BNF, (2) the conditions in which Mo and P are co-limiting BNF,

and (3) the independent vs. interactive effects of anthropogenic air pollution and BNF on tree nutrition in boreal forests.

2.7. Acknowledgements

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Competing Interests

We have read and understood The Science of the Total Environment's policy on declaration of interests and declare that we have no competing interests.

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3. CHAPTER 2

Nitrogen and phosphorus deficiencies of planted black spruce trees following scarification as indicated by critical concentrations, nutrient ratios and diagnostic vector analyses on two sites in Northern Quebec.

Reference: Evaluation of nitrogen and phosphorus deficiency in *Picea mariana* of two climates in Northern Quebec, Canada. Forest Science. (*To be submitted*)

The present study looks at black spruce foliar concentrations of nitrogen and phosphorus on establishing plantations 18 years after soil scarification by disc trenching in two climate regimes of Northern Quebec.

The results showed that 18 years after scarification both nitrogen and phosphorus deficiency were reduced, but were still detected on sites in both climates. The effect of scarification on nitrogen and phosphorus foliar concentrations were lower in the more continental climate. In non-scarified plantations of both climates phosphorus was found to be more deficient than nitrogen.

Evaluation of nitrogen and phosphorus deficiency of *Picea mariana* in two climates in Northern Quebec, Canada

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3.1. Abstract

We tested the effects of scarification on nitrogen (N) and phosphorus (P) foliar concentrations of black spruce trees growing on plantations in two climate regimes of Northern Quebec, Canada. Black spruce trees were fertilized with either N or P in both scarified and non-scarified plots. Black spruce needles were collected on the two sites in mid-September 2016. Needles were measured for N and P concentrations and biomass. Critical levels, N:P ratios and diagnostic vectors were used to determine nutrient deficiencies. We showed that 18 years following soil scarification both N and P deficiency were reduced compared to the control. The overall effect of scarification was lower at the site in a more continental climate, compared to the site in a more maritime climate. P was more deficient than N across both climates on non-scarified plots. Our data underscore the importance of using multiple approaches to evaluate nutrient deficiencies. We also show that care should be taken when determining fertilization protocols for boreal forests on sites in differing climates, as their responses can be varied.

Keywords

Scarification; Black spruce; Nitrogen; Phosphorus; Diagnostic vector analysis; Boreal forest

3.2. Introduction

Mechanical site preparation through soil scarification is a common silvicultural practice used to promote regeneration establishment after natural or anthropogenic disturbances (Löf et al. 2012). In boreal ecosystems which are characterized by short growing seasons, slow decomposition rates, and inherently limited forest productivity, scarification is used to disturb the organic layer and expose the mineral soil prior to reforestation activities. The benefits of soil scarification on conifer growth in boreal ecosystems has been repeatedly observed, both in the short- (e.g. Hallsby and Örlander 2004; Henneb et al. 2015), mid- (e.g. Thiffault et al. 2010; Örlander et al. 1998) and long-term (e.g. Boateng et al. 2011). In northern latitudes, scarification increases soil temperature in the rooting zone, improves water availability to regenerating trees, reduces shrub dominance and competition for resources, creating suitable microsites for planted seedling survival and growth (Örlander et al. 1990, Prévost 1992). Typically, scarification also increases decomposition rates of organic matter with positive impacts on soil nutrient availability (Brand, 1991), an effect that could be similar to that of fertilization (Thiffault et al. 2017). However, the extent to which nutrient availability affects tree growth after scarification is not fully understood. Although significant growth responses are observed following scarification, we do not know the extent to which nutrient availability is improved. Moreover, regional climate conditions and soil types are expected to influence tree nutritional responses to scarification, as the precipitation and temperature regimes are the most important driver of soil fertility (Aerts and Chapin, 1999).

In the province of Quebec (Canada), a climatic gradient of temperature and precipitation exists. For example, the region of Côte-Nord located in northeastern Quebec, north of the Gulf of St. Lawrence, it has a maritime climate characterized by cold and wet annual conditions. This type of climate has been shown to have higher abundances of ericaceous shrubs. These

types of plants have long been known to have detrimental effects on forest tree species. For example, Yamasaki et al. (1998) found that black spruce seedlings (*Picea mariana* (Mill.) BSP) growing in proximity to *Kalmia angustifolia* L. had significantly lower concentrations of foliar N and P. Further inland in Quebec the region of Abitibi has a continental climate characterized by warm and dry average annual conditions. These distinct regional climates are known to bring about different soil conditions and understory communities (Thiffault et al. 2015). Since scarification generally increases soil N availability (Prévost 1992), we hypothesized that the relative N to P limitation of planted trees decreases in scarified plots, relative to non-scarified conditions. We also hypothesized that nutrient deficiencies are more pronounced in a cold and wet climate (Côte Nord site) than in a warm and dry climate (Abitibi site), notably because of the thicker soil organic layer, and higher abundances of ericaceous shrubs.

There are several approaches to quantify nutrient deficiencies of conifer growth. Comparing foliar concentrations of macronutrients to known critical levels is the simplest of them (Brakke, 1994). For example, minimum critical needle concentrations for black spruce are estimated at 12 and 1.4 mg g^{-1} for N and P, respectively (Swan, 1970). However, this approach has been criticized as it does not take into account nutrient stoichiometry, most importantly the ratio of N to P (Chapin 1980; Koerselmann and Meuleman 1996). This ratio approach has shortcomings it is possible that N or P, or both may limit growth even if a satisfactory N:P ratio is observed. Diagnostic vector analysis is a very useful approach that takes into account changes in nutrient concentration, nutrient content and biomass relative to a control (Timmer and Stone, 1979; Haase and Rose, 1995). By comparing the pre-fertilization state (i.e. Control) to a tree fertilized with a supposed limiting nutrient we can evaluate increase in nutrient concentration and needle biomass to determine the extent of nutrient limitation. Interpretation of this technique is more reliable when using single fertilizer additions as opposed to combinations of multiple nutrients (Valentine and Allen, 1990)

In this context, our objectives were to determine the extent to which scarification alleviates N and/or P limitations to growth for black spruce planted in boreal ecosystems, and determine if their effect on tree nutrition depends on the climate regime. To do so, we conducted a scarification × fertilization field experiment on two contrasting sites located 700 km apart along a climatic gradient in boreal Quebec (Canada). Therefore, determining if soil scarification can indeed alleviate nutrient limitations to black spruce growth, and hence, enable planted trees to express their full growth potential, has major implications to sustainable forest management.

3.3. Materials and Methods

3.3.1 Study Sites and Experimental Design

We conducted a field experiment at two sites in two climatic regions of Quebec. The first site (Côte-Nord) was north of the town of Baie-Comeau (49°47 18‴N, 69°17 12 ‴W) (see Thiffault and Jobidon, 2006 for full site description). In summary, mean annual precipitation in the region is 1300 mm and a daily average temperature of 13.2 °C from June to September. Black spruce trees were 140 cm +/- 83 cm. The second site (Abitibi) was near the town of Senneterre (48°83 37‴N, 76°86 40‴W) (see Thiffault et al., 2005 for full site description). In summary, mean annual precipitation in the region is 950 mm and daily average temperature of 15.0 °C from June to September. Black spruce trees were 185 cm +/- 4 cm. Both sites were cutovers that were converted into black spruce plantations using disk trenching scarification in the fall of 1999. Complete random block experimental design comprising 10 and 13 replicates in Abitibi and Côte-Nord, respectively. Each block was split into two (≥ 540 m^2) plots, which were randomly attributed a control treatment (no scarification) or a scarification treatment by

disk trenching. In June 2000, the plots were planted using containerized black spruce seedlings produced from local sources in governmental nurseries.

3.3.2. Field Sampling

In mid-May of 2016, 15 black spruce trees from scarified plots and 15 black spruce trees from non-scarified plots were selected at each site. Within a given plot we selected three trees to receive one of three treatments (+N, +P, and Control). Fertilized trees were amended with either 57 g of NH_4NO_3 or 53g of $(Ca(H_2PO_4)_2) * H_2O$. We applied the fertilizers uniformly over a 0.5 m radius around the tree base (i.e. equivalent to 240 kg N ha^{-1} and 120 kg P ha^{-1}). In late-September 2016, we harvested current-year needles from the top third of the tree crown around each tree to prevent bias from orientation to the sun. Needle samples were transported to the laboratory, where they were dried at 50 °C for 72 h.

3.3.3. Needle Elemental Analysis

Dried needles were counted in sets of 100 (i.e. 60 trees × 3 replicates). These needles were used for subsequent elemental analyses. We mixed the dried needle samples with liquid N_2 and ground them into a fine powder using a mortar and pestle. Ground subsamples (ca. 100 mg) of each of the 180 samples were encapsulated in tin foil and analyzed for total N using a Vario Macro Elemental Analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). The remaining material from each sample was pooled back together (from 180 to 60) and digested in trace metal grade HNO_3 diluted with MilliQ water. We analyzed the digests for total P using an X-Series-2 Inductively Coupled Plasma–Mass Spectrometer (Thermo scientific, Waltham, Massachusetts, United States) (Rodushkin et al., 1999).

3.3.4. Statistical Analyses

Within each scarification treatment and climatic region, we plotted a graphical vector analysis diagram based on the method described by Timmer and Stone (1979). These normalized values were used to plot vectors, which were tested using either content (x), concentration (y), or biomass (z) as dependent variables with linear-mixed models with fertilizer treatment as a fixed effect and block as a random effects variable (n=5). N and P concentrations and N:P ratios were tested using the same mixed model approach, with block as a random effect variable. We performed the analyses using the "lme4" package (Bates et al., 2015) of the R computing environment (R Core Team, 2013). Data were verified for normality and homoscedasticity using QQ-plots and residuals vs. fitted values plots. We used $\alpha = 0.05$ as a threshold to consider an effect as being significant, unless otherwise stated.

3.4. Results

3.4.1. N and P Concentrations and N:P Ratios

At the Côte-Nord site, fertilization significantly increased N and P concentrations, while scarification significantly increased P concentration ($p = 0.019$), and P concentrations were higher in the scarification treatment ($p = 0.03$). The N:P ratio was increased with N fertilization and decreased with P fertilization (< 0.001). At the Abitibi site both N and P concentrations significantly increased and the N:P ratio decreased with fertilizer P addition on non-scarified plots (Table 1).

Site	Treatment	Fertilizer	*N Conc.* (mg g^{-1}) (Δ%)	*P Conc.* (mg g^{-1}) (Δ%)	N:P 8.57 (Δ%)
Côte-Nord	Non-Scarified	C	9.28 b (-23)	1.10 b (-21)	8.44 b (2)
		+N	17.94 a (50)	1.55 ab (11)	11.57 a (35)
		+P	11.03 ab (8)	2.46 a (76)	4.48 c (-48)
	Scarified	C	10.02 b (-17)	1.15 a (-18)	8.71 a (2)
		+N	11.09 ab (-8)	1.25 a (-11)	8.87 a (4)
		+P	9.95 b (-17)	1.37 a (-2)	7.26 a (-15)
Abitibi	Non-Scarified	C	9.61 b (-20)	1.15 a (-18)	8.36 a (-2)
		+N	11.45 a (-5)	1.21 a (-14)	9.46 a (10)
		+P	10.43 ab (-13)	1.71 b (22)	6.10 b (-29)
	Scarified	C	9.66 c (-20)	1.27 a (-9)	7.61 a (-11)
		+N	10.92 ab (-9)	1.23 a (-12)	8.88 a (4)
		+P	10.16 bc (-15)	1.30 a (-7)	7.82 a (-9)

Site	Scarification	Fertilizer	Scarification x Fertilizer
Cote Nord (N Conc., P Conc., N:P)	0.06, 0.019, 0.92	0.003, 0.002, <0.001	0.15, 0.03, <0.001
Abitibi (N Conc., P Conc., N:P)	0.54, 0.38, 0.98	<0.001, 0.009, <0.001	0.76, 0.16, 0.19

Table 1. N concentration, P concentration and N:P ratio of Côte Nord and Abitibi on scarified and non-scarified plots. Different lower-case letters indicate significant differences between the fertilization treatments within site and scarification treatment. Δ% indicates percent changes relative to the critical concentrations of 12, 1.4 for N and P, respectively. This would also indicate an optimal N:P ratio of 8.57. ANOVA results are indicated below with N conc., P conc., and N:P ratio.

3.4.2. Vector Diagrams

The responses of the N and P concentrations and contents to the treatments differed between the two sites (Fig. 8 and Table 2). At the Côte-Nord site, both the + N and + P fertilization treatments increased the N and P concentrations and contents, but only in the non-scarified plots. In contrast, at the Abitibi site the fertilization treatments caused changes in both the scarified and non-scarified plots. Specifically, + N fertilization on non-scarified plots showed significant increases in needle biomass (Fig. 8 and Table 2).

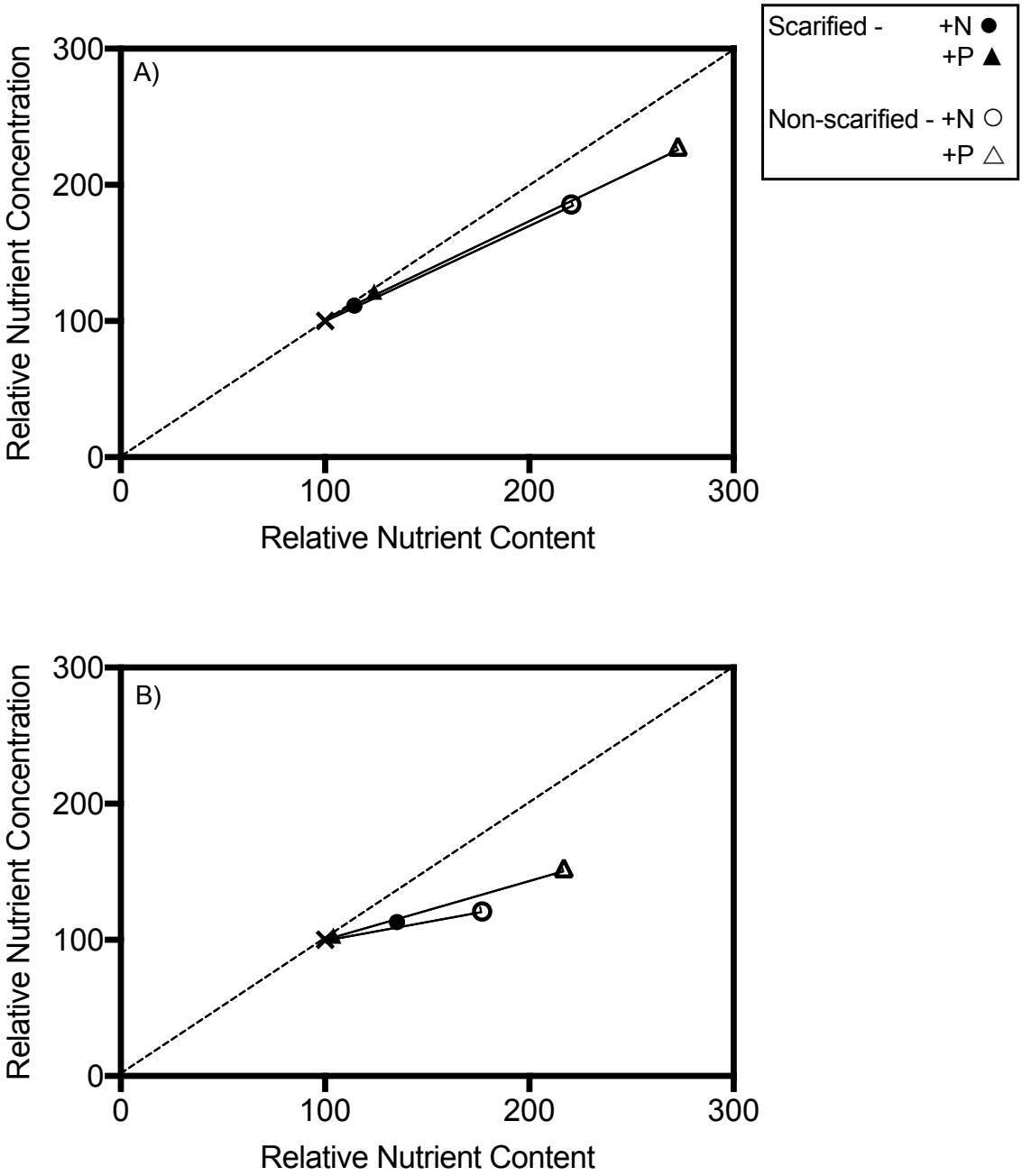

Fig. 8. A) Diagnostic vector analysis of needle nitrogen and phosphorus at the Côte-Nord site. B) Diagnostic vector analysis of needle nitrogen and phosphorus at the Abitibi site.

	Treatment	Fertilizer	Change in relative *mass*	Change in relative *cont.*	Change in relative *conc.*	Nutritional effect	Nutrient status	Possible diagnosis
Nitrogen diagnosis								
Côte-Nord	Non-scarified	+N	0	++++	++++	Accumulation	Non-toxic	Luxury consumption
	Scarified	+N	0	0	0		No change	
Abitibi	Non-scarified	+N	+	+++	+++	Accumulation	Limiting	Deficiency
	Scarified	+N	0	++	++++	Accumulation	Non-toxic	Luxury consumption
Phosphorus diagnosis								
Côte-Nord	Non-scarified	+P	0	++++	++++	Accumulation	Non-toxic	Luxury consumption
	Scarified	+P	0	0	0		No change	
Abitibi	Non-scarified	+P	0	++	+	Accumulation	Non-toxic	Luxury consumption
	Scarified	+P	0	0	0		No change	

Table 2. Summary of results from diagnostic vector analyses. + = $p < 0.15$ ++ = $p < 0.10$ +++ = $p < 0.05$ ++++ = $p < 0.01$

3.5. Discussion

The concept of foliar critical concentrations has been studied for decades, and has been used as a means to determine black spruce nutrient status (Watt and Heinselman, 1965; Swan, 1970). This information provides us with an idea of the optimal levels of each nutrient for black spruce growth. Swan (1970) found low levels of N to be 12 mg g^{-1}, with a critical concentration of 12-15 mg g^{-1} and an adequate level of 15 mg g^{-1}. The generally accepted critical concentration in spruce of 12 mg g^{-1} as indicated by Braekke (1994), for Norway spruce, would indicate that all non-fertilized trees were still N limited, even after scarification. However, with N fertilization this critical value was only attained on the Côte Nord non-scarified plots, indicating the highest uptake of N and therefore most severe N limitation. Swan (1970) found low P levels to be at 1.4 mg g^{-1}, a critical concentration between 1.4-1.8 mg g^{-1} and an adequate level of 1.8 mg g^{-1}. Therefore, the critical concentration is 1.4 mg g^{-1} and all unfertilized trees remain under the critical level. After fertilization, the greatest response in P concentration was observed on the Côte Nord non-scarified plots, indicating that there are higher nutrient deficiencies of both N and P at Côte Nord compared to Abitibi.

Even though critical concentrations provide an indication of the plant nutrient status of each nutrient individually, plants require nutrient in stoichiometric ratios. Currently there are no known values for optimal N:P ratio for black spruce. However, in Norway spruce, an optimal N:P ratio was found to be at around 6-7 by Clarholm and Rosengren-Brinck (1995), and at around 6-12 by Mellert and Göttlein (2012), corresponding to an optimal N:P ratio of 10:1 found for all terrestrial plants by Knecht and Goransson (2004). Based on this information all unfertilized trees are within the optimal range. However, both + N and + P treatments at Côte Nord on non-scarified plots deviate above and below these ranges respectively. Therefore, interpretations of N:P ratios should be evaluated within the scope of its individual components.

Both the critical value method and nutrient ratio method provide good initial ideas of nutrient limitation. However, they only take into account nutrient concentrations. Also incorporating changes in needle biomass, we can better predict long-term responses in stem wood growth (Timmer and Morrow, 1984). Addition of a deficient nutrient would cause both increased concentration of the nutrient in the needles and increased growth measured needle biomass. Therefore, a vector shifting up and to the right would indicate a limitation of either N or P.Vectors were consistently longer at the Côte Nord site indicating a larger nutrient deficiency in both N and P, relative to Abitibi. Taking into account both sites on non-scarified plots we notice that the P vectors are longer than N vectors. This indicates that there is more P limitation relative to N limitation. However, this difference disappears following scarification. The main benefit of this method compared to the ones described above is the ability to distinguish between luxury consumption and limitation, as a response from changes in needle biomass. Even though the increases in biomass were not always significant, we observed every vector to follow a directional shift that would indicate nutrient limitation of either N or P.

Each of the approaches have strengths and weaknesses, for this reason it is best to evaluate all approaches together to form a more comprehensive diagnosis. However, the methods do provide similar diagnoses. For example, nutrient limitation is higher at the Côte Nord site, in a maritime climate compared to the Abitibi site in a more continental climate. This could be due to the organic matter layer being over twice as thick at the Côte Nord site than Abitibi, as well as higher ericaceous shrub abundances at the Côte Nord site (Reicis et al., 2017). It has been shown that black spruce growing in proximity to ericaceous shrubs have lower foliar N and P values, therefore it is likely that higher ericaceous shrub abundances would cause a N and P deficit (Yamasaki et al., 1998). It has also been shown that with increasing organic matter layer depth availability of both N and P will decrease (Simard et al., 2007).

Each method provides the same general consensus that scarification may alleviate nutrient deficiencies, however not completely eliminate. Our recommendation is that methods should be combined and interpreted in conjunction with each other to provide the most informed diagnoses.

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4. DISCUSSION AND GENERAL CONCLUSION

The impact of anthropogenic influence in northern ecosystems has been increasing dramatically as human populations continue to grow and expand northward. This has further emphasized the need to understand the outcomes civilization is having on northern ecosystems. Boreal forest nutrient cycling is generally slow and understanding how the increased availability of nutrients will affect productivity should be a focus moving forward. Our results indicated that nutrient stress and silvicultural disturbance in boreal forests are factors that drastically alter nutrient cycling. These factors will ultimately have implications for our success in the understanding plant nutrition in boreal ecosystems.

In the first chapter, we show how nutrient stress, caused by deposition of phosphorus and heavy metals can alter nutrient cycling in local areas along roadsides by impacting BNF. As anthropogenic influences in boreal ecosystems continue to expand the stress on BNF may lead to repercussions on a much more global scale. The research presented in this chapter may be used to better inform nutrient cycling models so that the levels of deposition at which BNF and ultimately forest nutrient cycling are altered.

In the second chapter, we show how a widespread soil silvicultural disturbance, can have positive effects on soil nutrient availability of boreal ecosystems in different climates. This study underscores the importance of proper silvicultural management on sites within different climates. This study has also underscored the importance of a multiple approach method to evaluating nutrient deficiencies in boreal forests. Ultimately, this study could have implications for silviculturalists who seek to manage sites that are within different climatic

regimes. These better managed sites will be able to contribute to their sustainability and long-term growth.

The research performed here goes a long way to addressing some of the unknowns regarding boreal forest nutrient cycling, but more importantly, it has raised numerous questions to be addressed in the future. For example, the contributions of the individual compounds present within atmospheric deposition. Also, the conditions under which Mo and P are co-limiting BNF and the conditions under which N and P are co-limiting growth of boreal forest trees. Further research in the area of boreal forest nutrient stress and silvicultural disturbance should be conducted along larger spatial scales to find patterns present on a global scale. With this research and subsequent future research, we can then better inform biogeochemical models that predict anthropogenic impacts in many northern ecosystems. This research could also be used to inform timber harvesting models through having a more fundamental understanding of tree nutrition in these remote areas.

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